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Concurrent levels of 11-ketotestosterone in fish surface mucus, muscle tissue and blood

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Summary

A rapid, reproducible method is described for extracting and comparing levels of the ether-soluble fish androgen 11-ketotestosterone (11-KT) in blood serum, muscle tissue and surface mucus. Because widely different volumes of extract were recovered after centrifugation from the three sources, it was important to express androgen levels as pg 11-KT/mg of total soluble protein (TSP). For six male and four female sexually-staged freshwater Koi (*Cyprinus carpio*), the method yielded similar pg 11-KT/mg TSP ratios in blood serum and extracts of muscle tissue and surface mucus, with the strongest correlation between blood serum and surface mucus. While male Koi were distinguishable from females based on the magnitude of 11-KT levels, reproductive stage and gonadosomatic index levels were not correlated with the 11-KT levels of either sex. Similar pg 11-KT/mg TSP ratios were also found for autologous muscle tissue and surface mucus extracts of 37 captured and sexed wild marine fishes representing seven genera. However, high 11-KT levels were not restricted to mature males. Collectively, results suggest that surface mucus collection (followed by 11-KT assay) is a useful alternative to more invasive methods of determining systemic hormone levels in fish. Without knowledge of seasonal variation in levels of this and other sex hormones, however, reliance on 11-KT levels alone may lead to spurious identification of gender, let alone reproductive stage.

Introduction

The androgenic steroid 11-ketotestosterone (11-KT) is associated with the process of spermiation, development of secondary sexual characteristics, and regulation of male reproductive behavior in most teleost species (Kime and Manning, 1982; Fostier et al., 1983; Koldras et al., 1990; Borg, 1994; Sullivan et al., 1997; Fine et al., 2004). In fisheries research, or in aquaculture, knowledge of systemic levels of 11-KT may be useful for revealing reproductive state and/or gender; however, such assays typically rely on invasive methods for obtaining samples, such as blood collection or muscle tissue biopsy. These methods can place undue stress on fish (Donaldson, 1990; Arukwe and Goksøyr, 1998), and often result in sacrifice for successful sample collection.

In the present study, we investigated a less invasive methodology for determining 11-KT levels in fish through comparative studies of levels in autologous blood serum, muscle tissue and surface mucus using fish of known reproductive status and/or gender. Our primary objective was to evaluate the extent to which 11-KT levels measured in surface mucus corresponded to those in blood serum and muscle tissue. This was a necessary step for ultimately gauging the utility of measuring 11-KT levels in surface mucus as a means of separating genders and/or determining the stage of maturity in: (1) live specimens without subjecting them to the stresses of muscle tissue and/or gonadal biopsy; or (2) eviscerated carcasses of unknown gender. In the process, it was necessary to resolve the problem of differential dilution/concentration of mucus-borne 11-KT at time of sampling (i.e. because of differing quantities of ambient water retained or evaporated during mucus sample collection).

During these comparative studies, elevated 11-KT levels were found among several immature and female fish as well as males. These results corroborate earlier reports (Leatherland et al., 1982; Slater et al., 1994; Cuisset et al., 1995; Lokman et al., 1998) that, in some species, the androgen is not a male-specific steroid hormone, and may have other functional roles.

Materials and methods

Ten sexually-active Koi (*Cyprinus carpio* L.) were obtained from a commercial aquaculture operation (Summerland Tropical Fish Farms, Homestead, FL, USA): six were males with an average total length (TL) of 28.3 cm and average weight of 358.6 g and four were females averaging 31.0 cm TL and 605.8 g. Each fish was bled by cutting a gill arch with a razor blade, collecting the blood, and separating the serum from the clot by centrifugation at 5°C. From 0.2 to 0.5 ***g*** of muscle tissue was biopsied from the caudal musculature and, with a sterile, rounded, stainless steel spatula, surface mucus was obtained by lightly scraping the skin directly above the muscle biopsy location. Care was taken to not contaminate the surface mucus with blood or muscle tissue. The gender of each Koi was validated by gross visual examination of gonadal tissue whereby each fish was characterized as male, female or immature. Next, with the aid of a microscope, the reproductive stage of each Koi was determined histologically from gonad sections preserved in 10% formalin (Barbieri et al., 1994; Lowerre-Barbieri et al., 1996; Grier, 2002).

To further compare levels of 11-KT in muscle tissue and surface mucus, a selection of wild fishes was collected from a variety of marine habitats off Marathon Key, Florida, using

cast net, spear gun and hook-and-line fishing. Immediately upon collection, samples of surface mucus and muscle tissue were obtained from each fish (as described above), placed in plastic containers, and transported on ice to the laboratory where they were then stored at −80°C until tested by the methods described below. Gender of wild fishes was determined via gross visual examination of gonadal tissue.

Prior to androgen assays, samples were thawed at 5°C and 0.1 g of muscle tissue and 0.5 ml of surface mucus scraped from the same fish were placed separately at the bottom of an 8 ml, glass tissue grinder (Kontes Glass Co., Vineland, NJ). The preparations were hand homogenized (30 strokes) with a glass pestle after adding 1 ml of 20 mM Tris-buffered NaCl (0.9%), pH 7.4 at 5°C, then centrifuged at 15 000 ***g*** for 15 min. The pellet was discarded and the supernatant fluid was saved for protein and 11-KT determinations. The blood serum was treated the same as the surface mucus for 11-KT determinations.

Protein was determined by the BioRad DC protein assay (Bio-Rad Laboratories, Hercules, CA). For 11-KT, 0.6 ml of the supernatant fluid was extracted two times with diethyl ether (Burkick & Jackson, Muskegon, MI) (7 ml, then 2 ml) at 25°C. Further extraction of the aqueous phase and interphase layers with additional diethyl ether did not yield additional 11-KT. The pooled ether fraction was poured off into a glass tube with a Teflon-lined cap and dried with a jet of dry N_2 in a fume hood under static conditions until the solvent was evaporated to dryness (approximately 20 min). The 11-KT was determined with an enzyme immunoassay EIA Kit (Cayman Chemical Co., Ann Arbor, MI) following the instructions of the manufacturer. All samples were quantified in duplicate, and the values were expressed as averages. For 11-KT, the standards ranged from 7.8 to 1000 pg ml^{-1}. The reproducibility of the EIA for 11-KT was confirmed by evaluating the intra- and inter-assay variability. The latter were calculated as a percentage of the coefficient of variation (CV) (100 × SD/mean), for replicate measurements of serum spiked with 11-KT. The quantity of the spiked hormone (pg ml^{-1}) was selected at the midpoint of values of the respective standard curves (approximately 50% hormone bound). The intra-assay variability for 11-KT in EIA, CV 8.7% (n = 36); the inter-assay variability for 11-KT in EIA, CV 8.9% (n = 8). The hormone assays were done blind of the gender.

To examine possible relationships between 11-KT, reproductive stage and gonadosomatic index (GSI), an additional 16 male and 13 female Koi were obtained from the same aquaculture operation. The sizes of these fish were approximately the same as the 10 Koi described above. Surface mucus was collected from each fish, extracted with diethyl ether, the extract was prepared as described above, and the levels of androgen were calculated as pg 11-KT/mg total soluble protein (TSP). The sex and stage of maturity were determined by visual examination of gonads of each fish, and by histological methods using 10% formalin-fixed preparations of gonad. The GSI was calculated as 100 × gonad weight (g)/body weight (g).

Results

Shown in the upper panels of Fig. 1 are average levels (+1 SE) of pg 11-KT/mg TSP in the blood serum (a), muscle tissue (b) and surface mucus (c) of six male and four female Koi. Histological analyses indicated that these six males were at the mid- to late-maturation stages, with abundant sperm in the gonads. The four females were all staged as gravid with hydrated oocytes measuring 35–80 μm in diameter (10 oocytes were measured per individual fish). Analysis of variance followed by *t*-tests indicated that mean 11-KT values for males were significantly higher ($P < 0.001$) in all cases. The greatest difference (i.e. 13-fold higher in males) in mean levels

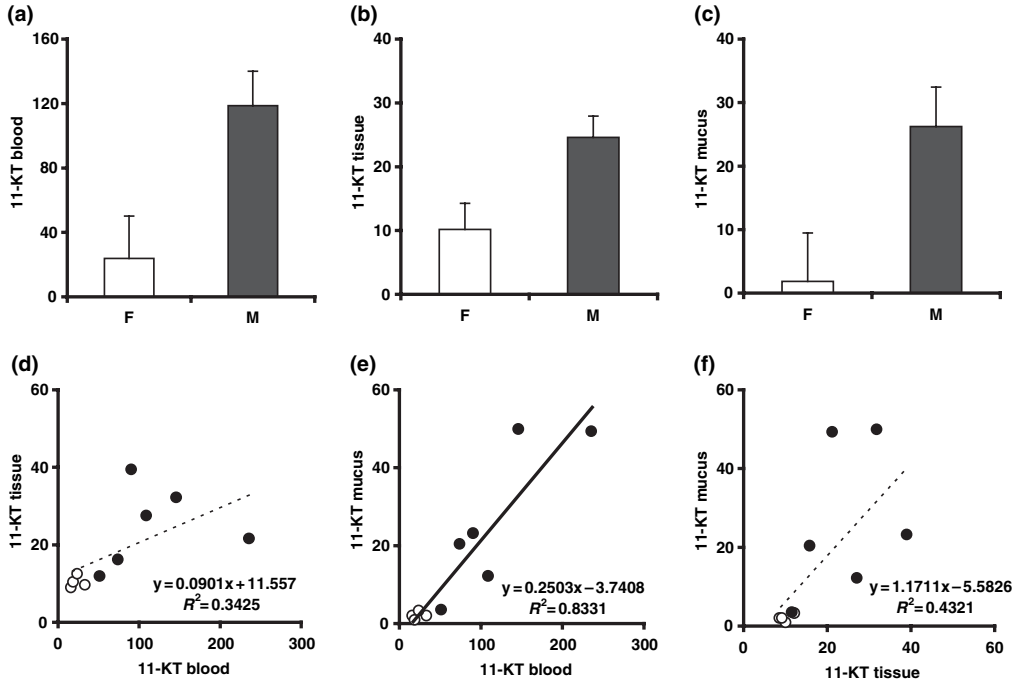

Fig. 1. Mean + 1 SE of pg 11-KT/mg total soluble protein from the sexually-active male (black bars) and female (white bars) Koi in (a) blood serum; (b) muscle tissue; and (c) surface mucus. Shown in lower panels are correlations between 11-KT levels in (d) blood serum and muscle tissue; (e) blood serum and surface mucus; and (f) muscle tissue and surface mucus. Black and white dots indicate male and female specimens, respectively

between sexes was detected in surface mucus. The lower panels of Fig. 1 illustrate the nature and strength of correlations between 11-KT levels in the blood serum and muscle tissue (d), blood serum and surface mucus (e), and muscle tissue and surface mucus (f). The strongest correlation was between blood serum and surface mucus ($R^2 = 0.83$).

Presented in Fig. 2 are levels of pg 11-KT/mg TSP in tissue extracts and autologous surface mucus of the 37, randomly-selected, wild marine fishes examined: nine spotted seatrout, *Cynoscion nebulosus* (four females, five males); six ballyhoo, *Hemiramphus brasiliensis* (4 ♀, 2 ♂); two sea bream, *Archosargus rhomboidalis* (1 ♀, 1 immature); one white mullet, *Mugil curema* (♂); three redfin needlefish, *Strongylura notata* (2 ♀, 1 ♂); six great barracuda, *Sphyraena barracuda* (4 ♀, 2 ♂), six gray snapper *Lutjanus griseus* (2 ♀, 3 ♂, 1 immature), two pinfish, *Lagodon rhomboides* (immature), and two bluestriped grunt, *Haemulon sciurus* (immature). Overall, the androgen levels in extracts of muscle tissue and surface mucus were similar; however, it is important to note that not all of the high 11-KT levels corresponded to male fish. Whereas a male spotted seatrout (no. 5) and male redfin needlefish (no. 20) had the highest pg 11-KT/mg TSP levels, the next highest levels belonged to a female redfin needlefish (no. 19) and an immature gray snapper (no. 32).

Figure 3 presents correlation analysis results for levels of surface mucus 11-KT vs gonad developmental stage (a, b) and GSI (c, d). Surface mucus levels of 11-KT were higher for males (a, c) than females (b, d), but 11-KT levels were predictive of neither reproductive stage ($R^2 = 0.006$–0.0496) nor GSI ($R^2 = 0.0516$–0.0614).

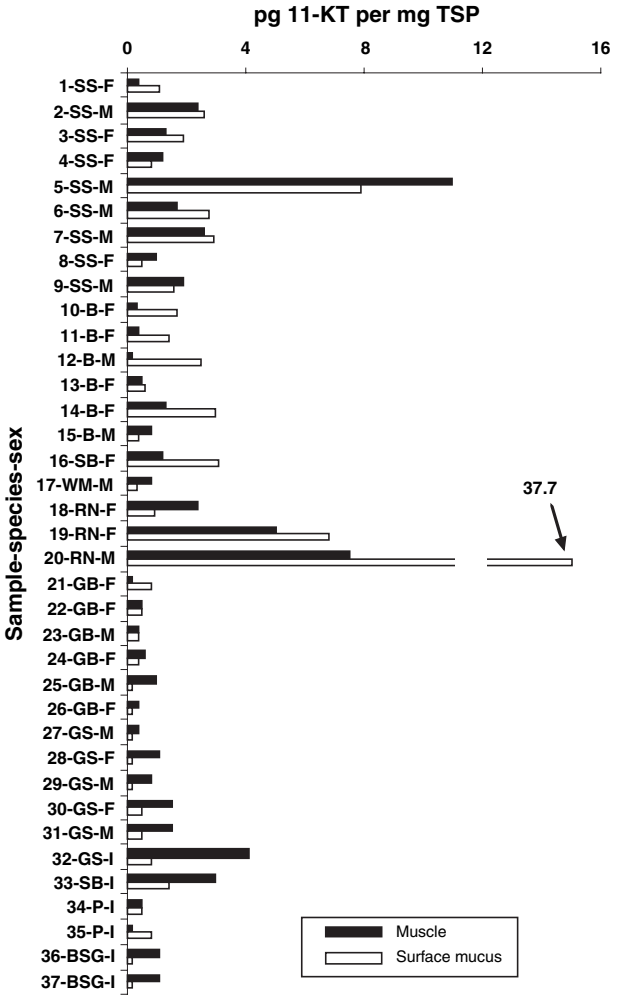

Fig. 2. Comparison of pg 11-KT/mg total soluble protein in muscle tissue (■) and autologous surface mucus (□) of 37 individual marine fishes representing seven different genera. The gender of each fish (M, male; F, female) was validated by gross visual examination of gonadal tissue, or, when necessary, in the laboratory with the aid of a dissecting microscope. Fish species codes (see text for Latin names) refer to: spotted seatrout (SS), ballyhoo (B), sea bream (SB), white mullet (WM), redfin needlefish (RN), great barracuda (GB), gray snapper (GS), pinfish (P) and bluestriped grunt (BSG)

Discussion

The primary purpose of these experiments was to compare relative levels of pg 11-KT in surface mucus with those in autologous blood serum and/or muscle tissue extract. These comparisons first required that 11-KT levels be expressed as ratios of TSP because unknown quantities of ambient water are simultaneously retained at time of surface mucus sample collection, or lost because of evaporation. As widely different volumes of extract are typically obtained from such different sources, consideration of hormone : soluble protein ratios is recommended, especially when comparing sex hormone levels in surface mucus with those from blood serum and/or muscle tissue.

Our results comparing autologous blood serum, surface mucus and muscle tissue from male and female Koi as well as those comparing surface mucus and tissue from 37 wild saltwater fishes indicate that, using our methods, all of these substances may be used for the determination of 11-KT. However, while collection and assay of 11-KT in surface mucus is feasible for those interested in less invasive methods than biopsy or blood collection for monitoring levels of this

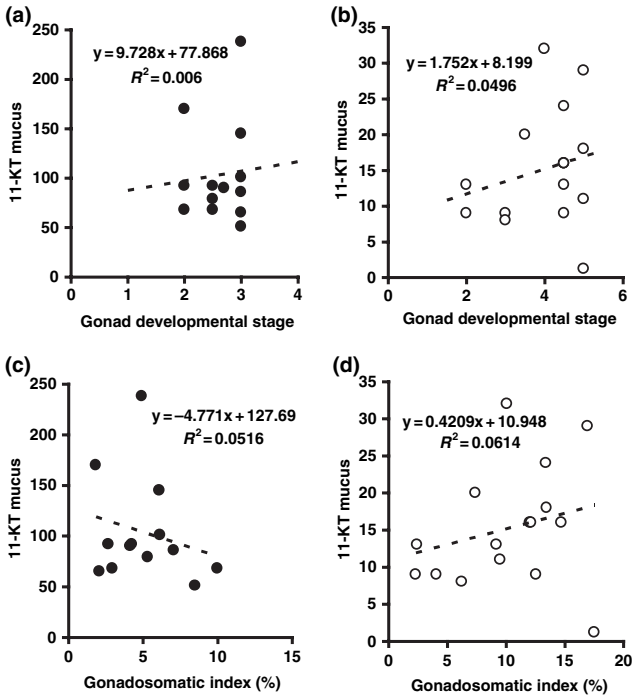

Fig. 3. Scatter plots and correlations of reproductive stage and gonadosomatic index vs levels of pg 11-KT (per mg total soluble protein) in autologus surface mucus of Koi. (a) Observed developmental stages of testes; (b) observed developmental stage of ovaries; (c) gonadosomatic index (GSI) of males; and (d) GSI of females. Black and white dots indicate male and female specimens, respectively

hormone in captive or recently-collected specimens, due caution is warranted if researchers rely on 11-KT levels alone for determination of gender or reproductive stage. Our data in Fig. 2 (fish nos 14, 16, 19, 32, 33) and the data of other investigators (Leatherland et al., 1982; Slater et al., 1994; Cuisset et al., 1995; Lokman et al., 1998) have shown that in some fishes, 11-KT is not a male-specific steroid hormone, and may have functional roles other than spermatogenesis. These include preparing maturing fish for spawning migrations (Lokman et al., 1998), secondary sexual characteristics (Leatherland et al., 1982; Borg et al., 1992), and stimulation of growth of heart and red muscle to increase swimming endurance (Thorarensen et al., 1996).

The literature is replete with examinations of relationships between GSI and 11-KT levels. However, the strength of these relationships appears to depend on the species of fish and time of sampling, probably also reflecting that the GSI does not reliably indicate specific maturation stages. Our experiments (Fig. 3) showed no correlation between 11-KT levels and GSI, and additionally, no correlation between 11-KT levels and gonad developmental stage in either male or female Koi. However, the Koi available for this investigation of surface mucus as a reliable non-invasive source of androgen did not include specimens in very early or late stages of gonad development. In a study of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*), Amer et al. (2001) showed that elevated serum levels of 11-KT were associated with an increase of the GSI, but levels continued to rise as the GSI declined with the initiation of active spermiogenesis. The data of Weltzien et al. (2002) showed highest GSIs and 11-KT plasma levels at stage IV of germ cell development of male Atlantic halibut (*Hippoglossus hippoglossus* L.), but both fell abruptly at stage V. Koldras et al. (1990) investigated sperm production and steroidogenesis in testes of *C. carpio*; their data revealed that GSI, in general, correlated well with other indices of maturation, including sperm production and area of the cysts. However, steroid production was not correlated with these indices of maturation. Similarly, a study on freshwater eels (*Anguilla dieffenbachia* and *A. australis*) found no clear relation between plasma 11-KT levels and GSI (Lokman et al., 1998). Although spermatogenesis in eels (*A. japonica*) was shown *in vitro* to be mediated by 11-KT (Miura et al., 1991a), a relationship between the proportion of spermatocytes and 11-KT levels described for artificially matured eels (*A. anguilla*) by Khan et al. (1987) did not exist for longfinned (*A. dieffenbachia*) or shortfinned (*A. australis*) male eels (Lokman et al., 1998) or for artificially-matured *A. japonica* (Miura et al., 1991b). Note that none of the above studies used surface mucus as a source of androgen.

Other laboratories have investigated surface mucus as a source of vitellogenin, the precursor of egg yolk proteins (Gordon et al., 1984; Kishida et al., 1992; Moncaut et al., 2003). Vitellogenin has been detected in surface mucus of females, but males possess functional copies of the gene, and its expression can be induced by environmental stressors and xenoestrogens (Donaldson, 1990; Arukwe and Goksøyr, 1998; Moncaut et al., 2003). Therefore, for certain practical applications, reliance on vitellogenin assay results is not without its limitations.

In conclusion, although surface mucus collection and assay is a viable, minimally-invasive means of quantifying 11-KT in fish, knowledge of 11-KT levels alone can be misleading. Androgens, including 11-KT, have been shown to vary seasonally (Fine et al., 2004) and this may have been a factor in the present study. Further research is warranted to reveal whether dual assays targeting both 11-KT and vitellogenin in surface mucus are more definitive for separating genders and/or determining the stage of maturity of live fish or eviscerated carcasses.

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References

Amer, M.A.; Miura, T.; Miura, C.; Yamauchi, K., 2001: Involvement of sex steroidhormones in the early stages of spermatogenesis in Japanese Huchen (*Hucho perryi*). Biol. Reprod. **65,** 1057–1066.

Arukwe, A.; Goksøyr, A., 1998: Xenobiotics, xenoestrogens and reproduction disturbances in fish. Sarsia **83,** 225–241.

Barbieri, L. R.; Chittenden, M. E. Jr; Lowerre-Barbieri, S. K., 1994: Maturity, spawning, and ovarian cycle of Atlantic croaker, *Micropogonias undulatus*, in the Chesapeake Bay and adjacent coastal waters. U.S. Fish. Bull. U.S. **92,** 671–685.

Borg, B., 1994: Androgens in teleost fishes. Comp. Biochem. Physiol. **109C,** 219–245.

Borg, B.; Antonopoulou, E.; Andersson, E.; Carlberg, T.; Mayer, T., 1992: Effectiveness of several androgens in stimulating kidney hypertrophy, a secondary sexual character, in castrated male three-spined stickle backs, *Gastrosteus aculeatus*. Can. J. Zool. **71,** 2327–2329.

Cuisset, B.; Fostier, A.; Williot, P.; Bennetau-Pelissero, C.; Le Menn, F., 1995: Occurrence and in vitro biosynthesis of 11-ketotestosterone in Siberian sturgeon, *Acipenser baeri* Brandt maturing females. Fish Physiol. Biochem. **14,** 313–322.

Donaldson, E. M., 1990: Reproductive indices as measures of the effects of environmental stressors in fish. Am. Fish. Soc. Symp. **8,** 109–122.

Fine, M. L.; Johnson, M. S.; Matt, D. W., 2004: Seasonal variation in androgen levels in the oyster toadfish. Copeia **2004,** 235–244.

Fostier, A.; Jalabert, B.; Billard, R.; Breton, B.; Zohar, Y., 1983: The gonadal steroids. In: Fish Physiology, Vol. 9A. W. S. Hoar, D. J. Randall, and E. M. Donaldson (eds). Academic Press, New York, pp. 277–372.

Gordon, M. P.; Owen, T. G.; Ternan, T. A.; Hildbrand, L. D., 1984: Measurement of a sex-specific protein in skin mucus of premature coho salmon (*Oncorynchus kisutch*). Aquaculture **43,** 333–339.

Grier, H. J., 2002: The germinal epithelium: its dual role in establishing male reproductive classes and understanding the basis for indeterminate egg production in female fishes. Proc. Gulf Carib. Fish. Inst. **53,** 537–552.

Khan, I. A.; Lopez, E.; Leloup-Hatey, J., 1987: Induction of spermatogenesis and spermiation by a single injection of human chorionic gonadotropin in intact and hypophysectomized immature European eel (*Anguilla anguilla* L.). Gen. Comp. Endocrinol. **68,** 91–103.

Kime, D.E.; Manning, N. J., 1982: Seasonal patterns of free and conjugated androgen in the brown trout, *Salmo trutta*. Gen. Comp. Endo. **48,** 222–231.

Kishida, M.; Anderson, T. R.; Specker, J. L., 1992: Induction of Vitellogenin in striped bass (*Morone saxatilis*): characterization and quantification in plasma and mucus. Gen. Comp. Endocr. **88,** 29–39.

Koldras, M.; Bieniarz, K.; Kime, D. E., 1990: Sperm production and steroidogenesis in testes of the common carp, *Cyprinus carpio* L., at different stages of maturation. J. Fish Biol. **37,** 635–645.

Leatherland, J. F.; Copeland, P.; Sumpter, J. P.; Sonstegard, R. A., 1982: Hormonal control of gonadal maturation and development of secondary sexual characteristics in coho salmon, *Oncorhynchus kisutch*, from lakes Ontario, Erie and Michigan. Gen. Comp. Endocrinol. **48,** 196–204.

Lokman, P. M.; Vermeulen, G. J.; Lambert, J. G. D.; Young, G., 1998: Gonad histology and plasma steroid profiles in wild New Zealand freshwater eels (*Anguilla dieffenbachia* and *A. australis*) before and after onset of the natural spawning migration. I. Females. Fish Physiol. Biochem. **19,** 325–338.

Lowerre-Barbieri, S. K.; Chittenden, M. E. Jr.; Barbieri, L.R., 1996: The multiple spawning patterns of weakfish, *Cynoscion regalis*, in the Chesapeake Bay and Middle Atlantic Bight. J. Fish. Biol. **48,** 1139–1163.

Miura, T.; Yamauchi, K.; Takahashi, H.; Nagahama, Y., 1991a: hormonal induction of all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). Proc. Natl. Acad. Sci. USA. **88,** 5774–5778.

Miura, T.; Yamauchi, K.; Nagahama, Y.; Takahashi, H., 1991b: Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. Zool. Sci. **8,** 63–73.

Moncaut, N.; Lo Nostro, F.; Maggese, M. C., 2003: Vitellogenin detection in surface mucus of the South American cichlid fish *Cichlasoma dimerus* (Heckel, 1840) induced by estradiol-17β. Effects on liver and gonads. Aquat. Toxicol. **63,** 127–137.

Slater, C. H.; Schreck, C. B.; Swanson, P., 1994: Plasma profiles of the sex steroids and gonadotropins in maturing female spring Chinook salmon (*Oncorhynchus tshawytscha*). Comp. Biochem. Physiol. **109A,** 167–175.

Sullivan, C. V.; Berlinsky, D. L.; Hodson, R. G., 1997: Reproduction. In: Striped bass and other *Morone* culture. Developments in aquaculture and fisheries science, Vol. 30. R. M. Harrell (ed.). Elsevier Science B.V., Amsterdam, The Netherlands, pp. 11–73.

Thorarensen, H; Young, G.; Davie, P. S., 1996: 11-Ketotestosterone stimulates growth of heart and red muscle in rainbow trout. Can. J. Zool. **74,** 912–917.

Weltzien, F.-A.; Taranger, G. L.; Karlsen, O.; Norberg, B., 2002: Spermatogenesis and related plasma androgen levels in Atlantic halibut (*Hippoglossus hippoglossus* L). Comp. Biochem. Physiol. (A) **132,** 567–575.

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